

NOTES

Development of a Multiplex Real-Time PCR for Detection and Differentiation of Malignant Catarrhal Fever Viruses in Clinical Samples[▽]

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Received 19 May 2009/Accepted 22 May 2009

A multiplex real-time PCR was developed using a single pair of primers and fluorescent probes specific for five malignant catarrhal fever viruses and an internal positive control. The assay was able to simultaneously detect and differentiate the viruses in clinical samples with high sensitivity (97.2%) and specificity (100%).

Malignant catarrhal fever (MCF), a lymphoproliferative syndrome primarily of ruminants, is caused by gammaherpesviruses included in the MCF virus group (5, 15). MCF viruses (MCFV) exist in nature as unapparent infections in well-adapted hosts but cause an often fatal disease in certain clinically susceptible species (20). Within the MCFV group, six viruses are clearly associated with clinical disease: ovine herpesvirus-2 (OvHV-2) (16, 18, 23, 24, 28), alcelaphine herpesvirus 1 (AIHV-1) (20, 21), caprine herpesvirus-2 (CpHV-2) (3, 8, 14), an MCFV of unknown origin causing disease in white-tailed deer (MCFV-WTD) (9, 11), ibex MCFV (MCFV-ibex) (17), and AIHV-2-like virus (10). MCF is increasingly being recognized as the cause of significant economic losses in several major ruminant species as well as a threat to certain susceptible species held in mixed-species confinement (6, 13, 18).

The diagnosis of MCF can still pose a challenge to clinicians and pathologists, even though the classical clinical signs and the histopathology are highly suggestive (18, 22). To confirm a diagnosis, several PCR assays have been used (1, 2, 4, 7, 25,

26); however, none of them is capable of simultaneously differentiating among MCFV, and several reactions have to be performed until the diagnosis can be established, which is time-consuming and expensive.

In this work, a multiplex real-time PCR that used one pair of primers in conjunction with fluorescently labeled probes specific for OvHV-2, CpHV-2, MCFV-WTD, MCFV-ibex, and AIHV-1 was optimized and validated for the identification of these pathogenic MCFV in clinical samples using a single reaction. AIHV-2-like virus was not included in the present study due to the unavailability of its sequence information and clinical samples.

The finding of a polymorphic region in the viral DNA polymerase gene containing unique sequences for each virus of interest, used as probe targets, flanked by conserved regions was a critical step in the development of the assay (Fig. 1). The presence of the flanking conserved sequences allowed a single pair of primers to amplify the expected 80-bp fragment from the DNA polymerase gene from all viruses of interest. This characteristic represented a great advantage to assay optimi-

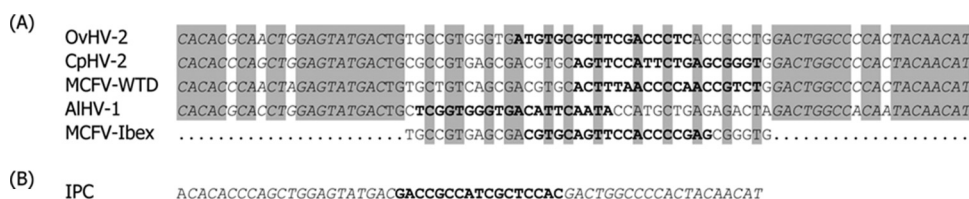


FIG. 1. (A) Alignment of the 80-bp sequences from the DNA polymerase genes of five MCFV known to cause disease in ruminants. Conserved nucleotides among sequences are highlighted, and the primer and probe target sequences are indicated in italic and bold, respectively. GenBank accession numbers are DQ198083 for OvHV-2, AF283477 for CpHV-2, and AF005370 for AIHV-1. The sequence from MCFV-WTD was available from our previous studies, and the sequence from MCFV-ibex was obtained in this study. (B) IPC oligonucleotide sequence.

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[▽] Published ahead of print on 3 June 2009.

TABLE 1. Primers and probes used for the multiplex real-time PCR

Primer or probe	5'-3' sequence and label(s) ^a
dpol771-F primerCACACCCAAGTGGAGTATGAC
dpol831-R primerATGTTGTAGTGGGGCCAGTC
OvHV-2 probeFAM-ATGTGCGCTTCGACCCCTC-BHQ1
CpHV-2 probeHEX-AGTTCCATTCTGAGCGGGT-BHQ1
MCFV-WTD probeTexas Red-ACCTTAACCCCAACCGTCT-BHQ2
AIHV-1 probeCy5-TCGGTGGGTGACATTCAATA-BHQ2
MCFV-ibex probeCy5-CGTGCAGTTCCACCCGAG-BHQ2
IPC probeTye705-GACCGCCATCGCTCCAC-BHQ2

^a FAM, 6-carboxyfluorescein; BHQ, black hole quencher; HEX, hexachlorofluorescein.

zation because interaction between primers was minimized. A synthetic internal positive control (IPC), consisting of an oligonucleotide of 58 bp containing the primer sequences flanking an irrelevant sequence used for specific probe binding (Fig. 1B and Table 1), was included in the assay as an indicator of the presence of PCR inhibitory factors in the reaction mixtures. The probes were labeled with fluorescent dyes with different emission spectra to allow simultaneous detection in the multiplex format (Table 1). Due to the limit of five channels in the real-time PCR system used, the probes for AIHV-1 and MCFV-ibex were labeled with the same fluorophore (Cy5); both probes were routinely tested simultaneously, and when a positive result for Cy5 was obtained, the sample was retested using the two probes separately.

During assay optimization, the concentrations of primers,

probes, and IPC in the reaction mixtures, which resulted in no interference among reagents and better assay sensitivity, specificity, and reproducibility, were determined by checkerboard titration using reference plasmids, containing the amplified 80-bp fragment of each virus, as templates. DNA amplification and detection were performed in a CFX96 real-time PCR detection system (Bio-Rad) using a 20-μl reaction volume containing 10 μl of Express qPCR SuperMix Universal (Invitrogen); 200 nM of each dpol771-F and dpol831-R primer (Table 1); 80 nM of each OvHV-2, CpHV-2, and MCFV-WTD probe; 8 nM of AIHV-1 and/or 320 nM of MCFV-ibex probes; 8 nM of IPC probe; 5.5×10^4 copies of the IPC oligonucleotide; and 100 ng of sample DNA, a variable concentration of reference plasmid DNA, or water. A nontemplate control and a positive control, a pool of all five reference plasmids, were included in each run, and all samples were tested at least in duplicate. The cycling protocol was 50°C for 2 min and 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 45 s with a plate read after each cycle. The PCR results were analyzed using CFX Manager software (Bio-Rad), and a threshold cycle of ≤ 40 was considered positive. Following assay optimization, thresholds were consistently adjusted to 298 relative fluorescence units (RFU) for 6-carboxyfluorescein, 248 RFU for hexachlorofluorescein, 94 RFU for Texas Red, 100 or 50 RFU for Cy5 (AIHV-1 or MCFV-ibex probe, respectively), and 45 RFU for Tye705, which resulted in higher specificity without losing sensitivity when samples with known virus status were tested.

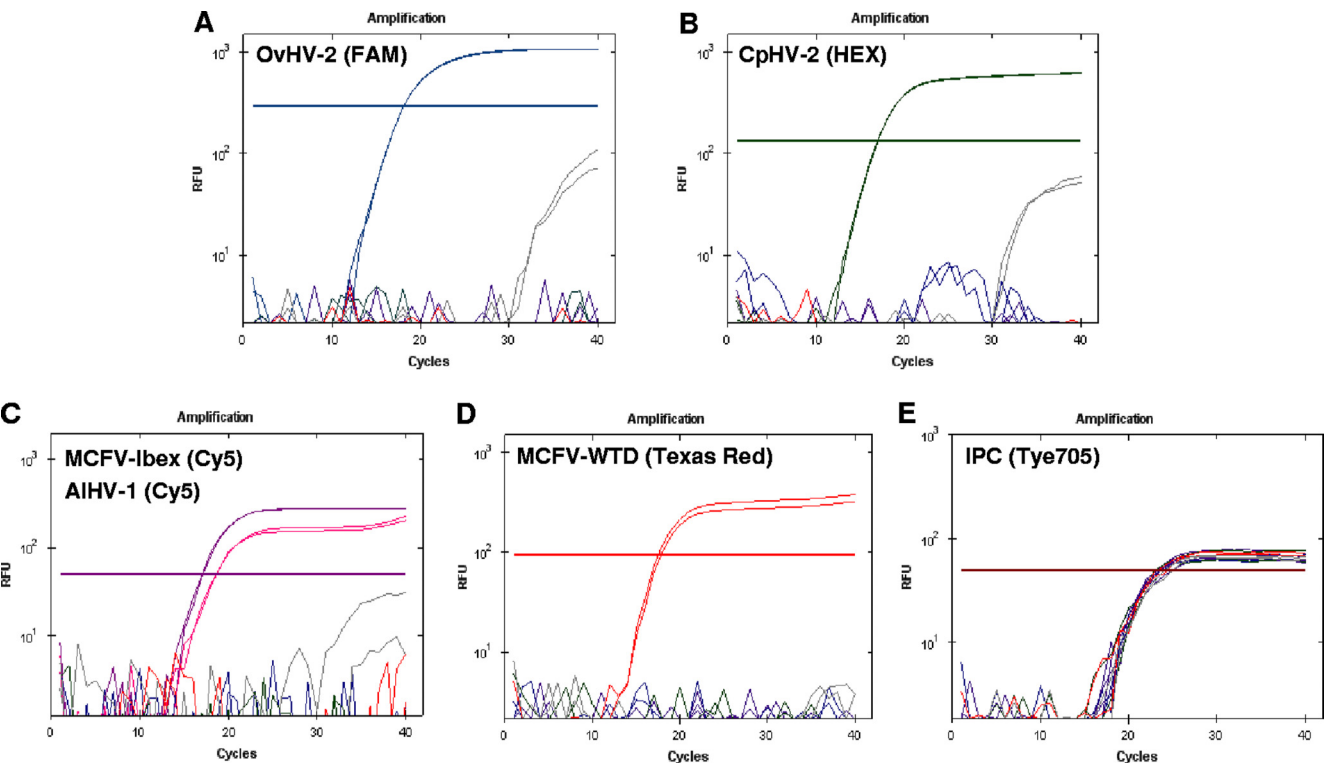


FIG. 2. Specificities of the probes used in the multiplex real-time PCR against reference plasmids. The charts show the amplification curves for the templates detected by the OvHV-2 probe (A), CpHV-2 probe (B), MCFV-ibex and AIHV-1 probes (C), MCFV-WTD probe (D), and IPC probe (E). Samples containing the reference plasmids for OvHV-2, CpHV-2, MCFV-WTD, AIHV-1, and MCFV-ibex and the no-template control are shown in blue, green, red, purple, pink, and gray, respectively. Horizontal lines represent the threshold established for each fluorophore.

TABLE 2. Results of the multiplex real-time PCR for MCFV in clinical samples with known virus status

Multiplex real-time PCR result	Gold standard ^a							Total
	No. of positive samples					No. of negative samples ^b		
	OvHV-2	AIHV-1 ^c	CpHV-2	MCFV-WTD	MCFV-ibex	Negative for other herpesviruses	Positive for other herpesviruses ^d	
Positive	81	3	10	6	4	0	0	104
Negative	3	0	0	0	0	47	12	62
Total	107					59		166

^a Clinical signs, histopathology, and PCR results.^b Clinical signs and histopathology not compatible with MCF and OvHV-2 nested PCR negative results.^c AIHV-1 cell cultures.^d Bovine herpesvirus 1 and 4, bovine lymphotropic herpesvirus, and other herpesviruses recently identified in ruminants.

The analytical sensitivity of the multiplex PCR, determined by using serial dilutions of a known copy number of each reference plasmid DNA, showed that all probes were able to detect as few as 50 copies of the specific viral DNA per reaction (data not shown). As demonstrated in Fig. 2, the probes were highly specific (Fig. 2), and no cross-reactivity among the fluorophores was observed. Using both multiplex and singleplex formats and each reference plasmid as a template revealed that the assay was reproducible, as observed by the low threshold cycle standard deviations obtained between each probe in both formats (0.1 to 0.4) and among four replicates (0.08 to 0.5).

The potential application of the assay to detect and differentiate OvHV-2, CpHV-2, MCFV-WTD, MCFV-ibex, and AIHV-1 was evaluated in clinical samples from animals with and without MCF. A panel of clinical samples either positive or negative for the viruses of interest and representing 14 different tissues and a variety of host animals, including cattle, sheep, goats, bison, deer, and antelope, among others, was used in the study. Clinical samples were defined as positive or negative for MCF based on clinical signs presented by the animal, histopathology, and PCR examination, using either specific primers for each virus (11, 12, 27) or consensus primers for herpesviral species (27), followed by sequencing for virus identification. Because AIHV-1 is classified as a select agent in the United States, clinical samples positive for AIHV-1 were unavailable and DNA from AIHV-1 cell cultures (Minnesota isolate, WC-11, and C-500) were used instead. By following these criteria, 166 samples were selected and classified as positive ($n = 107$) or negative ($n = 59$) for MCFV.

All 59 negative samples, which included 47 clinical samples with histopathology inconsistent with MCF and negative for OvHV-2 by PCR plus 12 samples negative for the virus of interest, but positive for other herpesviruses of ruminants (15), were also negative on the multiplex PCR (Table 2), defining an assay specificity of 100%. Among the samples previously tested as positive, 81 of 84 were confirmed by the multiplex PCR as positive for OvHV-2, whereas all samples known to be positive for CpHV-2 ($n = 10$), MCFV-WTD ($n = 6$), MCFV-ibex ($n = 4$), and AIHV-1 ($n = 3$) were also positive by the multiplex PCR. The presence of inhibitors in the PCR was detected in four known positive samples as determined by negative results for the IPC as well as for the virus. These samples were diluted (1:10) and retested, and positive results for the virus and for the IPC were obtained. Three of 84 samples positive for

OvHV-2 resulted in false negatives. The reason(s) OvHV-2 DNA was not detected by the multiplex PCR in these samples is unknown; the level of viral DNA in the samples, as determined by OvHV-2 real-time PCR (25), was above the analytical sensitivity of the assay, and because the IPC was detected, there was no apparent PCR inhibition in the reactions. In any case, it is recommended to evaluate negative results in conjunction with clinical signs and histopathology, and when a false-negative result is suspected, other confirmatory tests, such as nested PCR, must be performed. Considering all five MCFV together, the multiplex real-time PCR had 97.2% sensitivity, which was comparable to the existing OvHV-2 nonnested and real-time PCR assays, which show sensitivities of 98 and 97%, respectively, when testing clinical MCF samples (25).

It is important to note that the multiplex PCR was designed to detect the presence of MCFV in samples of clinically affected animals, when the viral DNA copy number is expected to be elevated in tissues and blood (19, 24). While the multiplex assay was suitable for detecting as few as 50 copies of each virus per reaction, whether this analytical sensitivity is enough to detect the virus in nonclinical samples still needs to be tested and evaluated.

In summary, the multiplex real-time PCR described in this study represents a rapid, reliable, and differential method for the identification of five pathogenic MCFV in clinical samples, which is of fundamental importance for the diagnosis of MCF. This assay is especially useful for the identification of the virus causing clinical MCF in animals from zoos and game farms with mixed-species operations where specific viruses need to be quickly differentiated and a plan for control established. Notably, the assay has great flexibility regarding the way it can be multiplexed, i.e., the probes included in the reaction mixture may be adjusted depending on the capabilities of the thermocycler used or according to the interests of different laboratories.

This work was supported by USDA/ARS CWU 5348-32000-024-00D.

We thank Janice Keller, Lori Fuller, and Shirley Elias for excellent technical assistance and Lindsay Oaks, Douglas Call, and Timothy Baszler for critical review of the manuscript.

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